Blood cytokine profile quantitative characteristics evaluation for identifying infection risk group in pigs

Alexander Agarkov[1*], *Lushnikova Tatiana*[1], *Nikolay* Agarkov[1], *Sivalneva* Diana[1], and *Irina* Nekrasova[1]

[1]Stavropol State Agrarian University, Serova st., 523, 355019 Stavropol, Russia

Abstract. Diseases in newborn animals cause significant damage to animal husbandry. This is a complex problem, in which, along with such factors as the environment and the pathogen, an important role is played by the reaction of the body of newborns and their close connection with the mother's body. The study of enzyme relationships in the functional system «mother-fetus-newborn» can make a significant contribution to solving the problem of improving the safety of the population of newborn animals. Newborn animals have different degrees of functional maturity. Functional capacity of some organs and the system of the newborn, in comparison with the parent individuals, can be determined both genetically and by the conditions of intrauterine development. Currently, a sufficient number of facts have been accumulated that any deviations or violations of homeostasis parameters the mother's body affects the fetus and vice versa. The main role in compensating for impaired functions belongs to the mother's body, but the fetus is also able to participate in these reactions to a certain extent. Functional integration of fetal and maternal homologous systems when performing homeostatic functions concerns the activity of the blood enzyme component. The aim of our research was to study quantitative and qualitative changes in the activity of blood enzymes in animals aged from 27 to 204 days.

1 Introduction

The revealed differences in the specificity of the embryonic isoantibodies formation are significant and apparently depend on the specifics of the methods and specific conditions for determining such specificity [1-8, 16]. In this case, an immunological defect may affect the activity of individual subpopulations of lymphocytes and macrophages, the ability of T-cells to produce soluble mediators or the ability of target cells to respond to these mediators.

Studies of the morphology of lymphoid tissue with immunological areactivity in the functional system "mother-fetus-newborn", the study of the mechanisms of reactions in vivo and in vitro are essential for elucidating the causes of isosensitization [4, 9-12, 17].

[*] Corresponding author: agarkov_a.v@mail.ru

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Based on the results obtained, we have developed scientifically based methods for assessing the degree of sensitization of mothers by fetal antigens of animals during pregnancy and measures to study fetoplacental conditions of fetal development. The latter determine the intensity of growth, development and the state of stability of the offspring in the postnatal period of ontogenesis [9, 13-15].

When the placental barrier function is impaired, the nature of immunobiological relationships in the mother-fetus system changes and the female's body is immunized with fetal antigens. The mother's isoantibodies, getting into the fetal circulation, can negatively affect the condition of the developing fetus. The probability of placental transfer of maternal isoantibodies (antibodies) to the fetus is much higher [2, 4, 5].

The revealed deviations of embryogenesis from the norm are the total expression of the indirect and direct action of the allogeneic factor. The latter effect is the most specific, since it always indicates direct damage to the fetoplacental complex [6,7, 11].

Since the reactivity of the organism in general and immunological reactivity in particular is an important property of the animal organism, any changes in it are mainly protective in nature. Therefore, in order to ensure higher viability, it is necessary to select parental pairs taking into account their immunological reactivity.

2 Research materials and methods

Experimental studies were conducted on piglets of a Large White breed on the pig breeding complex LLC "SVK", collective farm Velikorodny of the Stavropol territory. Histological studies were performed in the histological laboratory of the Scientific Diagnostic and Therapeutic Veterinary Center of the Stavropol State Agrarian University.

Biochemical parameters were determined on laboratory equipment in the “Scientific Diagnostic and Medical Veterinary Center” of the Stavropol State Agrarian University.

The experiments were carried out on 22 piglets. Control and test groups of animals aged from 27 to 204 days were divided in five age groups: I - 27 - 46 days, II - 45 - 53 days, III - 82 - 91 days, IV - 114 - 202 days, V - 193 - 204 days. There were 11 animals in the control and in the test group.

Blood samples from animals were obtained in the morning hours before feeding from the ear vein into polypropylene tubes containing a blood clotting activator. Quantitative activity determination of aspartate aminotransferase (AST), alanine aminotransferase (SGPT), glutamyltranspeptidase (GTP), total lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) was detected on a biochemical automatic analyzer ACCENT-200 with the help of reagent kits by Cormay (Poland).

The cytokine profile included: interleukin content - 1β (IL-1β), interleukin - 2 (IL-2), interleukin - 4 (IL-4), interleukin -10 (IL-10), tumor necrosis factor -α (TFN-α), γ-interferon (IFN-γ), which was determined by enzyme-linked immunosorbent assay, (ELISA) with subsequent consideration of the results on the «Uniplan-TM» spectrophotometer in accordance with the approved guidelines for diagnostic kits. Complex porcine recombinant cytokines of type I- alpha and type II- gamma, and a mixture of alpha and gamma interferons were studied as well.

The cytokine profile of the blood was evaluated as follows: heparin-stabilized blood was collected from experimental and control groups of piglets. Then the stabilized blood was diluted 1:1 PBS within 2 hours after sampling and added to test tubes Leucosep™, containing 60 % of the density gradient FICOLL-PAQUE™ Plus, to isolate the mononuclear profile of peripheral blood cells. The remaining erythrocytes, which are often present after isolation in the blood in newborn pigs, were lysed with a buffer Gibco™ ACK. The cells were placed in 96-well plates with 0.5×10 6 cells/well in RPMI 1640 medium (Gibco®) with 10% fetal serum. After 1 hour of incubation, the cells were

stimulated with the same LXR agonists as in the adjuvant with isoantigens: agonist TLR 1/2 (10 mcg/ml Pam3Cys L2000 from the microcollection EMC), agonist TLR 7/8 (5 mcg/ml R848, Resiquimod by InvivoGen), agonist TLR 9 (5 mcg/ml sequence CpG ODN-type A D32, 5'-ggTGCGTCGACGCAGggggg-3', by Eurofins Genomics.), mixture of agonists TLR 1/2, 7/8 and 9 (with 10 mcg/ml, 5 mcg/ml and 5 mcg/ml respectively), mixture of agonists TLR 1/2 and 9 (10 mcg/ml and 5 mcg/ml respectively) or the cells were left unstimulated as a negative control. Single TLR agonists of the following combinations were used for the criterion assessment of the cytokine profile of immunological reactivity: TLR 1/2 + TLR 7/8 + TLR 9 и TLR 1/2 + TLR 9.

For intracellular staining of mononuclear cells, they were repeatedly stimulated with isoantigens. During the last 4 hours of stimulation, brefeldin A (BD Bioscience) was added into each well to inhibit release and to ensure intracellular detection of cytokines. A supernatant for leukocyte activation (containing monomycin and forbol-12-myristate-13-acetate) (PMA) with BD GolgiPlug™ (BD Biosciences) was used as a positive control in accordance with the manufacturer's instructions, and as a negative control, the samples were not stimulated with brefeldin A.

All pigs, except the control group, received primary isoimmunization at the age of 3 days, followed by repeated isoimmunization at the age of 21 days. Non-immunized animals were injected intramuscularly with 1.0 ml of PBS. Isoantigens (1.0 ml) were injected intramuscularly into the medial side of the right pelvic limb in the form of a patch. The patches were removed after 24 hours. Heparin-stabilized blood samples (approximately 15 ml) were obtained on days 1, 3, 7 and 21 for analysis IFN-γ ELISpot or flow cytometry (FCM).

After isoimmunization, the injection site had been monitored for 4 days for a local reaction. In the isoimmune reaction, we evaluated redness and swelling of the skin on a scale developed by us, from 0 to 3 (unchanged) for each pelvic limb (the maximum overall score is 6). Specific IgG antibodies in serum samples were evaluated using indirect enzyme-linked immunosorbent assay of antibodies (Ingezim PRRS 2.0) in accordance with the manufacturer's instructions. The sample ratio was considered positive when equal to or greater than 0.4.

The specific cellular immune response was evaluated using a solid-phase immune analysis kit (ELI Spot analysis) (Porcine IFN-γ ELISpot PLUS (ALP) by Mabtech). Then the percentage of intracellular cells staining TNF or IFN-γ in subpopulations of T cells and NK cells was determined. On the 21st day of life in non-immunized piglets, the selected blood was analyzed for the average ratio of subpopulations of T cells, NK cells and B cells in the general population of the mononuclear series.

Cytokine production by subpopulations of T cells and NK cells was determined using a 4-stage staining protocol using 6 colors. First, the cells were incubated with a stain BD Horizon™ Fixable Viability Stain 450 (FVS450 by BD Biosciences) in accordance with the manufacturer's instructions. The cells were then incubated with directly labeled PE-Cy7 mouse antigens CD3ε (BB23-8E6-8C8 clone by BD Biosciences) (PG164A clone, WSU, Pullman, WA, USA). After surface staining, the cells were fixed in 4% paraformaldehyde and after washing with 0.1% saponin (Panreac Applichem), they were incubated with directly labeled Alexa Fluor 647® Anti-Human TNF-α (MAb11 clone by BioLegend), PE mouse-anti-swine IFN-γ (P2G10 clone by BD Biosciences) in 0,3% saponin followed by washing with another 0.1% saponin.

A marker for B cells. (P2G10 clone by BD Biosciences) in 0.3% saponin, followed by washing another 0.1% saponin, directly labeled Alexa Fluor 647® CD21 isoantibodies were added as an additional surface (BB6-11C9.6 clone by SouthernBiotech).

The cytokine profile was analyzed on FACSVERSE™ (BD Biosciences) using software BD FACSsuite™. Flow cytometry data was analyzed using Flowjo™ software version 10.0.

After that , the cells were classified according to the expression of the following combinations of surface markers (Gerner et al., 2015 ; Sinkora et al., 2013): T cells (CD3+), T helper (Th) cells (CD3 + CD4+), mature T cells (Tm) (CD3 + CD4 + CD8a+), Cytotoxic T cells (Tcyto) (CD3 + CD4 - CD8a+), NK cells (CD3 - CD8a +) and B cells (CD3 - CD21 +).

The proportion of different subpopulations was measured as a percentage (relative level) in the living population of peripheral blood mononuclears. For Th, Tm, Tcyto and NK cells, we determined the percentage of TNF or IFN-γ positive cells.

The obtained scientific data were processed using computer programs "Statistica 6.0" (Stat Soft Inc., USA) and Microsoft Excel (in the form of an arithmetic mean and a standard error of the mean (M±m). Comparisons of the obtained digital data from the aggregates were carried out by evaluating the normal distribution using the Student's t-test. The differences were considered statistically significant at $p<0.05$.

3 Results

Evaluation of the immune-reactive and cytokine profile of blood in newborn piglets in postnatal ontogenesis during alloimunization.

The dynamics in the formation of the immunological reactivity in piglets were evaluated by the intensity of the formation of anti-erythrocyte antibodies during alloimmunization.

The experiments were carried out on 22 piglets of the pig breeding complex LLC "SVK", collective farm Velikorodny. Control and test groups of animals aged from 27 to 204 days were formed in 5 age groups: I - 27 - 46 days, II - 45 - 53 days, III - 82 - 91 days, IV - 114 - 202 days, V - 193 - 204 days. There were 11 animals in the control and in the test group.

The formation of immune reactivity in piglets was determined by the production of antibodies to erythrocyte antigens. The donor to the recipients was selected according to the piglets' certification data using 9 erythrocyte antigen systems using 63 reagents manufactured at the Federal State Budgetary Institution «The Russian State Center for Animal Feed and Drug Standardization and Quality" and passed comparative tests.

The donor was a saw No. 5214 with relatively high immunogenicity, which had an antigen on erythrocytes. The piglets did not have this antigen and had to develop antibodies against it.

Piglets were immunized three times intramuscularly with whole citrate blood of a donor sow in a dose of 2 ml. The interval between injections was 7 days. Before immunization, piglets' blood serum was examined to detect natural anti-erythrocyte antibodies. On the 7th day after each injection, blood samples were taken, serum was isolated and the content of antibodies and their titer were determined. The intensity of the immune response in piglets of different age groups was assessed by the time of antibody production in blood serum and their titer.

Hemolytic tests of blood serum obtained from piglets before the experiment did not detect natural antibodies to erythrocyte antigens of the donor pig. There were no antibodies in the blood sera of piglets obtained 7 days after the introduction of the first donor blood and only 7 days after the second injection, hemolytic activity was manifested in the blood serum of some piglets (Table 4).

Table 1. Hemolytic activity of piglets' blood serum after the second immunization

Group	Age	Animal	Piglets'	Antibody titer during serum dilution

	group	number	age, days	H	2	4	8	16	32
Test	I	1079	27	0	0	0	–	–	–
Control		5674	27	0	0	0	–	–	–
Test		5649	38	44	44	0	–	–	–
Control		5651	38	0	0	0	–	–	–
Test		5650	39	0	0	0	–	–	–
Control		1059	39	0	0	0	–	–	–
Test	II	5624	45	44	44	0	0	–	–
Control		5625	45	44	44	0	0	–	–
Test		5622	46	44	44	0	0	–	–
Control		5627	46	44	44	44	0	–	–
Test	III	5541	82	0	0	0	0	–	–
Control		5539	82	0	0	0	0	–	–
Test		5535	85	44	44	44	0	–	–
Control		5532	85	44	44	44	0	–	–
Test	IV	5474	114	44	44	44	0	0	–
Control		5488	114	0	0	0	0	0	–
Test		5490	119	44	44	44	44	0	–
Control		5476	119	44	44	44	0	0	–
Test	V	5245	193	44	44	44	44	44	44
Control		5265	193	44	44	44	44	0	0
Test		5250	197	44	44	0	0	0	0
Control		5237	197	44	44	44	44	0	0

Note. The value 44 indicates complete lysis of the donor's erythrocytes with a two-fold reaction; 0 - absence of lysis

The serum of piglets of age group I, with the exception of one animal from the test group, did not show hemolytic activity, which indicates the immaturity of their immune system. Immunological reactivity was clearly detected in the serum of piglets of age groups II-V, and it increased with increasing age. However, some piglets of 3-4 months age (No. 5541 and No. 5539 from group 1, and No. 5488 from group IV) were unable to produce antibodies to the injected antigen.

The same pattern was observed after the third injection of the antigen, that is, 21 days after the start of immunization.

Piglets of the age group I, with the exception of two individuals, did not produce antibodies, at an older age the same animals were reactive as after the second administration of the antigen. Similar results were obtained in both cases. There was a discrepancy only in one case (after the third injection of the antigen, serum activity additionally appeared in one pig of age group V).

In some piglets, the formation of the immune system was delayed up to 3 months (No. 5541 and No. 5539) and even up to 4 months (No. 5488), which obviously indicates the existence of individual characteristics. The most intense reactions to the introduction of the antigen were observed in animals at an older age.

In support of the study, a clear local reaction to alloimmunization was established. A stable local skin reaction was observed in most experimental groups of piglets after the introduction of isoantigens by intradermal injections, characterized by variable local redness.

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However, in some groups of animals (at the age of 21 days), a clearer skin reaction (degree 2 and 3) was observed than after primary alloimmunization. This skin reaction, moderate to severe, was noticeable 5 days after alloimmunization, but completely disappeared two weeks after repeated alloimmunization. No local reaction was observed after intramuscular injection.

The cellular immune response after alloimmunization and control administration of isoantigens was assessed by the percentage of positive cells staining IFN-γ or TNF in various subpopulations of T cells (Th, Tm, Tcyto) and NK cells re-stimulated in vitro.

Single TLR agonists (TLR 1/2, 7/8, 9) and combinations of TLR agonists (TLR 1/2 + 9 and TLR 1/2 + 7/8 + 9) was used to stimulate neonatal peripheral blood mononuclears to determine their in vitro activity with respect to cytokine induction.

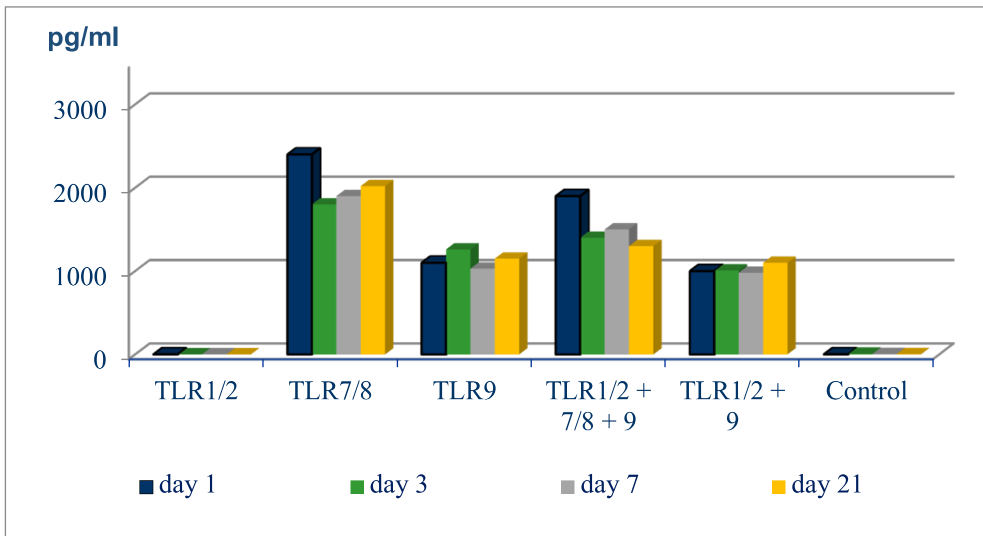

Fig. 1. Testing of supernatants on IFN-γ

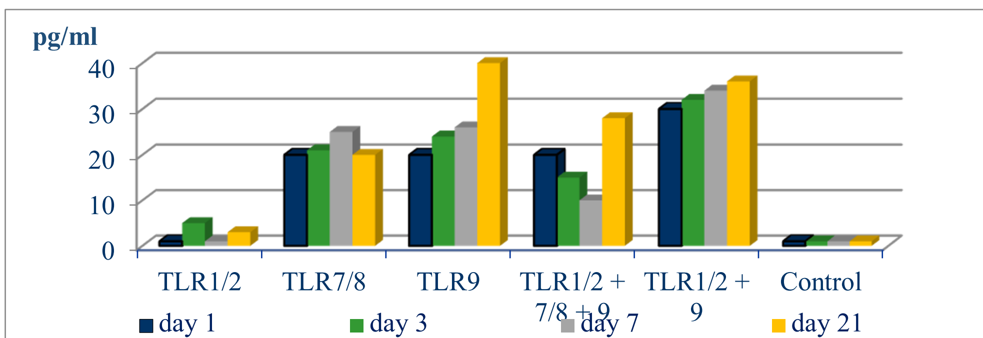

Fig. 2. Testing of supernatants on IFN-12p40

IFN-γ and IL-12p40 responses were mainly produced after TLR 7/8 and 9 stimulation and after stimulation with both combinations (TLR 1/2 + 9 and TLR 1/2 + 7/8 + 9), where the levels of IL-12p40 were high, but below the level of IFN-γ, compared with non-stimulated control samples (Figure 1 and figure 2).

High levels of IFN-α were observed only after TLR 9 stimulation and TLR 1/2 +9 combination. However, the response decreased when TLR 1/2 and TLR 7/8 were added to TLR 9 stimulation (Figure 3).

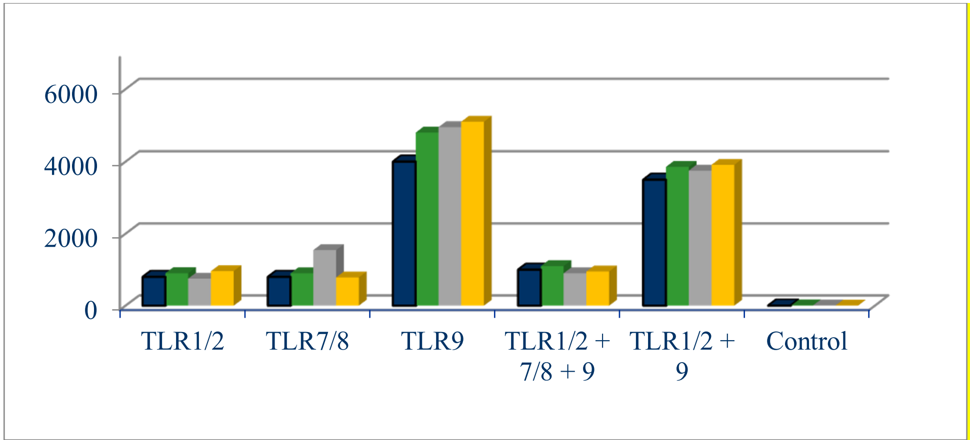

Fig. 3. Testing of supernatants on IFN-α

Cytokine response was evaluated after stimulation with isoantigenic TLR agonists in newborn pigs, 3-day-old piglets, 7-day-old piglets, 21-day-old piglets (n=22) with the establishment of various Toll-like receptor agonists (TLR 1/2, TLR 7/8 or TLR 9) and combinations of TLR agonists (TLR 1/2 + 7/8 + 9 and TLR 1/2 + 9). The supernatants were tested for IFN-γ, IL-12p40, IFN-α, IL-4, IL-1β, IL-6, IL - 10, IL-8 and TNF using Luminex multiplex.

We found that the overall IL-4 response was low, but significantly increased in the experimental group of piglets at various age periods, with the exception of TLR 1/2 stimulation (Figure 4).

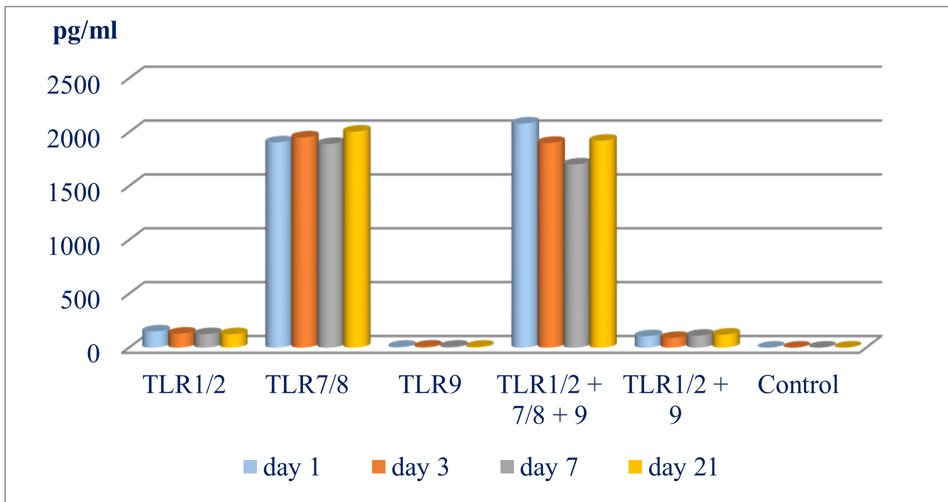

Fig. 4. Testing of supernatants on IL-1β

Significantly elevated levels of IFN-γ, IL-12p40, IL-4, IL-1β, IL-6 and IL-10 were induced in the presence of TLR 7/8 (Figure 5-7), compared with non-stimulated control samples.

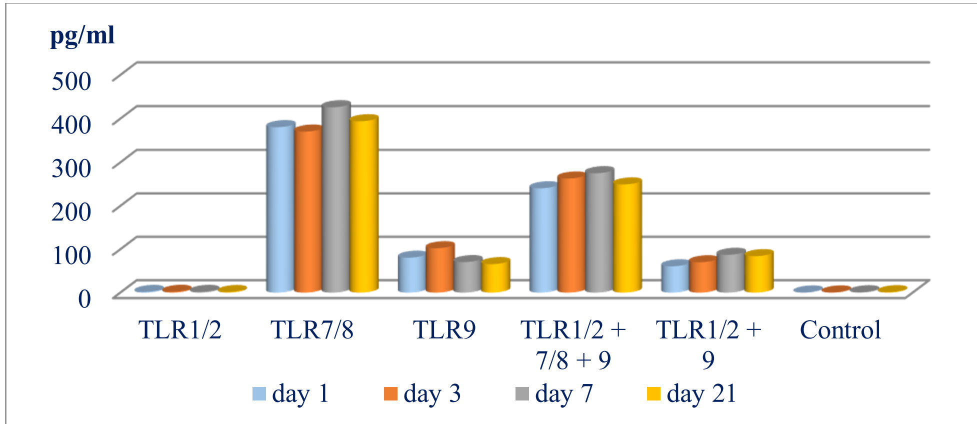

Fig. 5. Testing of supernatants on IL-6

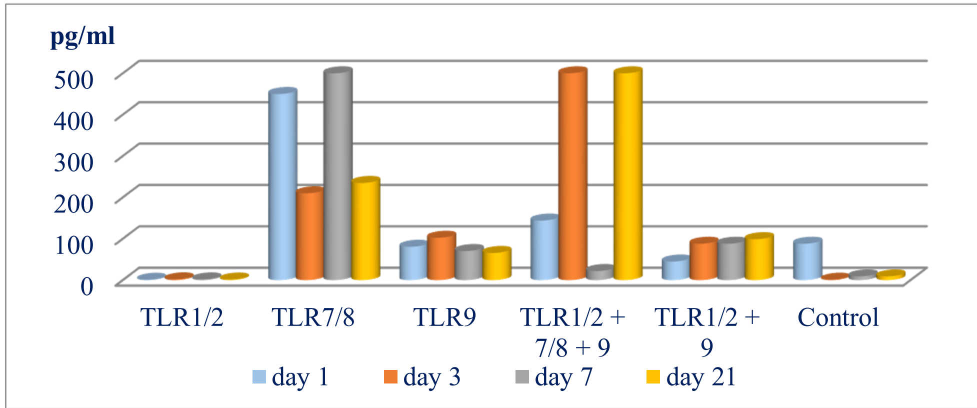

Fig. 6. Testing of supernatants on IL-10

It is noteworthy that only stimuli containing TLR 7/8 caused a significant increase in IL-1ß, IL-6 and IL-10. In contrast, the TLR 1/2 agonist was the least active of the three TLRs for the induction of this group of cytokines. Elevated levels of IL-8 were observed in a mixed type in the experimental groups.

In general, the combination of TLR agonists 1/2 + 7/8 + 9 stimulated the production of significant levels of IFN-γ, IL-12p40, IL-4, IL-1β, IL-6 and IL-10 in experimental animals, compared with unstimulated control samples, and the TLR 1/2 agonist showed minimal potential for induction of cytokine production, compared to TLR 7/8 and 9 agonists (Figure 6).

After alloimmunization, studies did not detect significant intracellular staining on TNF and IFN-γ after isoantigen stimulation in various subpopulations of T cells and NK cells in any of the measured groups.

Two weeks after repeated alloimmunization in the experimental groups, compared with intact animals, there was a significant increase in the percentage of IFN-γ-positive cells after alloimmunization in all subpopulations of T cells and NK cells.

The total percentage of cells with TNF staining (on average <0.05%) was significantly lower than with IFN-γ staining (on average 2.0–5.0%). Only the Th and Tm subpopulations showed a significant increase in the percentage of TNF-positive cells compared to the control group, as indicated by specific intracellular IFN-γ responses (Figure 7).

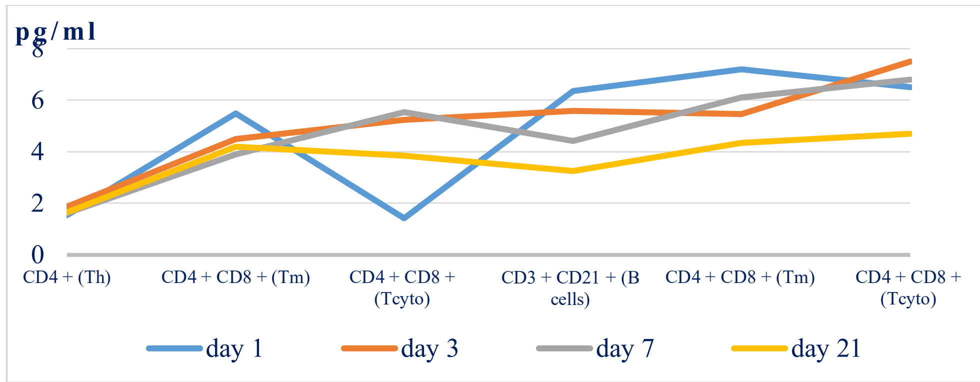

Fig. 7. Specific intracellular responses IFN-γ

Isoantigen-specific IFN-γ response after repeated in vitro stimulation in non-alloimmunized or alloimmunized sow against isoantigen using various adjuvants (ISA29, SWE, TLRa and SWE + TLRa) and cutaneous alloimmunization (skiSE + TLRa).

The percentage of positive IFN-γ cells in various subpopulations of T cells (CD4+ (Th), CD4 + CD8 + (Tm), CD4 – CD8 + (Tcyto)) and NK cells (CD3– CD8+) were analyzed 2 weeks after repeated alloimmunization one week after the control administration of isoantigen.

Seven days after the control administration of isoantigen, all alloimmunized animals showed a significantly lower percentage of IFN-γ-positive cells after stimulation in subsets of Th, Tm and Tcyto, compared with intact animals.This decrease was not significant for NK cells. No significant intracellular TNF responses, seven days after the introduction of isoantigens, were observed in any of the age groups.

It was found that the percentage (relative level) of various subsets of T cells (Tm and Tcyto), NK cells and B cells in non-stimulated antigens 7 and 21 days after the introduction of isoantigens, while the percentage of Tm cells increased significantly between 7 and 21 days after the introduction of isoantigen in the groups of animals studied.

4 Conclusions

The immunological reactivity of a newborn animal is largely determined by the state of the maternal organism and depends on the placental conditions of development during the fetal period. The fact of detection of allogeneic stimulation by embryonic antigens of offspring indicates a violation of placental conditions of development.

The concept of immunological areactivity includes a state of hypersensitivity as a special cell-mediated immunological method of intensive response. This concept generalizes pathological changes that can appear in any organ and any tissue during the induction of senesibilization of the maternal organism in relation to the developing fetus.

Tissue modification of the antigen before sensitization in some cases does not reduce the degree of subsequent reaction to the native antigen, and sometimes even enhances it, which is probably caused by the variety of progenitor effector cells, the specificity of which is different.

According to the results of the conducted studies, it was found that the degree of morphofunctional disorders is due to the immunological load of high titers of isoantibodies. To determine the areactive state of animals, it is necessary to use a set of interrelated immunobiological indicators reflecting the state of the mother and fetus, which reduces the risk of having offspring with signs of reduced viability.

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